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14. ABSTRACT This project ultimately aims to identify the role of tumor stroma in inducing prostate carcinogenesis. The hypothesis to be tested is that tumor stroma, in the form of carcinoma-associated fibroblasts (CAFs) isolated from human prostate cancer patient tissues, can initiate malignant transformation of human embryonic stem cells (hESCs). In the past year, we have spent a good deal of time refining the protocols and techniques that allow these experiments to be completed with high reproducibility and confidence that the fibroblastic cells being tested are of a malignant pathology. Using these refined techniques, we have generated a number of tissue recombinants using CAFs plus hESCs. We have also generated a number of positive and negative control recombinations. These grafts will be harvested in early April 2006. Overall, this project is progressing well, with some major technical hurdles overcome. Whatever the outcome of these experiments is, we will contribute significantly to the current understanding of the role of tumor stroma in the process of prostate carcinogenesis.					
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INTRODUCTION

This project ultimately aims to identify the role of tumor stroma in inducing prostate carcinogenesis. To date, the contribution of prostate tumor stroma to the carcinogenic process are equivocal; specifically whether the tumor stroma is involved in initiating, fueling the progression or both. This project seeks to test the hypothesis that tumor stroma, in the form of carcinoma-associated fibroblasts (CAFs) isolated from human prostate cancer patient tissues, can initiate malignant transformation in human embryonic stem cells (hESCs).

If this hypothesis is proven, then tumor stroma will be implicated in initiating prostate cancer and future work should attempt to dissect the contribution of specific stromal elements to initiation of tumorigenicity. In addition, we will provide evidence that the tumor stroma is a potential target for prostate cancer therapies, as well as generating a new model of human prostate cancer that will be available to test prevention strategies, and/or novel therapeutics. Alternatively, if this hypothesis is not correct, then this study will provide evidence that epithelial insult together with changes to the stromal environment lead to malignant transformation. Nonetheless, such information will greatly enhance our understanding of the carcinogenic process.

In the last 12 months, this project has experienced a number of technical problems. The main problem related to the quality of the CAFs that were isolated from prostate cancer patient tissues. The collection technique we initially used did not provide us with CAFs from confirmed malignant regions of the prostate. Secondary to that, we also experienced problems with the graft preparation methodology. Both of these issues have been addressed over the past 6-8 months and we have now set up a series of tissue recombinants using the refined protocols and these grafts will be harvested at the end of March 2006. We expect to have definitive results from these experiments by the middle of this year. The details of work towards each task are outlined below.

BODY

Task 1: Collection of normal prostatic fibroblasts (NPFs) and carcinoma-associated prostatic fibroblasts (CAFs) and establishment of hES cells [Months 1-6].

a. Patient recruitment and tissue collection from men with prostate cancer at radical prostatectomy, for collection of carcinoma-associated prostatic fibroblasts (CAFs).

As reported last year, during 2004 we obtained human ethics approval for the collection of carcinoma-associated fibroblasts (CAFs) from prostate cancer patients. We initially collected tissue from three patients using a blind biopsy technique. To do this, we palpated the tumor once the prostate was surgically removed and 10-15 biopsy samples were blindly taken from the estimated 'tumor region'.

We isolated fibroblasts from these tissue samples using the technique described in Olumi *et al.*, (1999). Previous reports indicate that primary cultures of CAF cells are indistinguishable from normal prostatic fibroblasts morphologically, immunocytochemically, and by growth characteristics (Olumi *et al.*, 1999), and therefore it was not possible to identify the phenotype (benign or malignant) of the fibroblasts in culture.

Following the isolation, we obtained pathology reports that we unable to confirm that the fibroblasts were isolated from malignant regions of the prostate; rather they were predominantly from benign regions. This was disappointing, but not surprising, since subsequent discussions with several pathologists indicate that this technique is typically poor at isolating malignant tissues; since the tumor is often small and localized, the chance of collecting tissue from adjacent non-malignant regions is fairly high. This was true in our experience.

Therefore, we have since taken the time to establish a technique where the pathologist is actively involved in the collection of tumor tissue pieces. We no longer use a blind biopsy technique, but instead the pathologist exposes the suspected tumor region and collects a tissue sample from the identified region. The pathology of the collected tissue is now confirmed using frozen sections of adjacent tissues by the pathologist at the time of collection.

This new technique has taken some time to establish, due to alterations to our current human ethics approval and collaboration with the pathologist, but the system is now in place and we have access to an extensive supply of CAFs from known malignant pathology as a result of excellent quality control measures. We have a unique relationship with our surgical and pathological colleagues that allow this to happen.

In the meantime, we worked closely with a collaborator from Vanderbilt University, Associate Professor Simon Hayward, who also uses prostate carcinoma-associated fibroblasts. He kindly provided us with enough CAFs to set up a round of tissue recombinants (described in **Task 2c**) before our new cell collections were in place.

b. Patient recruitment and tissue collection from men undergoing non-prostate related urological surgery in which there is no histopathological evidence of carcinoma of the prostate, for collection of normal prostatic fibroblasts (NPFs).

To date, we are still working towards completing Task 1b. We are currently using normal prostatic fibroblasts (NPFs) kindly provided by Associate Professor Simon Hayward.

We plan to isolate fresh NPFs when the pathologist is actively collecting CAFs from malignant tumors using frozen sections as confirmation; he will then collect non-malignant tissue from an adjacent region.

c. Establishment of culture of both normal prostatic fibroblasts and CAFs.

Despite the difficulty in collecting good quality fibroblasts from both malignant and normal tissues, we have managed to establish culture conditions that support prostatic fibroblast growth. Work towards this task is complete and will be applied to the new CAFs and NPFs as they are collected from now on.

d. Examination of growth characteristics and properties of CAFs compared to normal prostatic fibroblasts using immunohistochemistry.

This has been completed on existing fibroblasts, but results do not directly impact on the study since the cells are not from malignant pathologies; analysis will need to be repeated with the fresh collection of CAFs and NPFs.

e. Establishment and optimization of hES cell cultures on feeder layers to maintain hES cells in undifferentiated state.

This has been completed, and is ongoing, as reported last year.

Task 2: Recombination of hES cells with CAF cells [Months 6-18]**a. Continue growing hES cells and CAF cells in culture.**

This task has been completed successfully and is on-going in the laboratory.

b. Recombination in vitro using collagen gel technique, followed by subrenal grafting in athymic mice at 3 time points: 4 weeks (1 month), 12 weeks (3 months) and 36 weeks (9 months) to test neoplastic and metastatic capabilities.

A series of initial tissue recombinations were harvested early in 2005, as reported in the previous annual report. As described for **Task 1a**, the CAFs were not of malignant phenotype and therefore results from those grafts were not useful. Importantly, we found that the fibroblasts survived when grafted under the kidney capsule, but no glandular structures, arising from the hESCs were evident (see Figure 1).

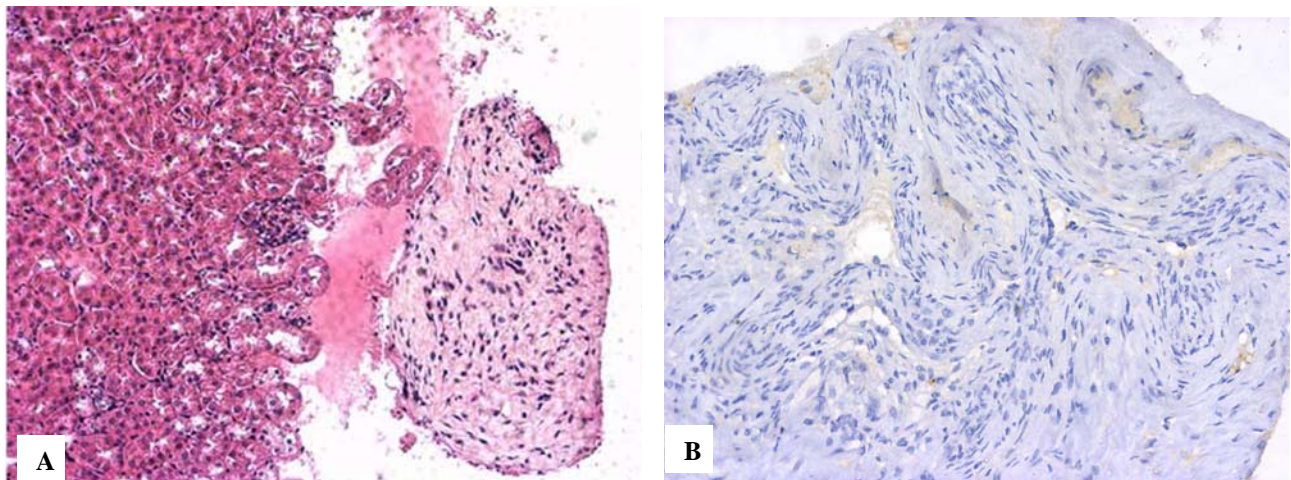


Figure 1: Fibroblasts+hESC tissue recombinants grafted under the kidney capsule of immune-deficient (SCID) mice for 8 weeks. A. H&E photomicrograph of fibroblasts and adjacent kidney tissue. B. Immunolocalisation of cytokeratin (CK) 8 (epithelial cell marker) showed no evidence of glandular tissue. Using the isolated fibroblasts (of benign origin), we showed that the fibroblasts survive at the graft site. Since these fibroblasts were not of malignant origin, tumor formation could not be assessed.

This could be due to a lack of differentiation signals from the benign fibroblasts, or maybe the hESCs did not survive the grafting process. In order to exclude the second possibility, we concentrated on optimizing the collagen gel grafting technique since this was the first time that we have grafted dispersed cells. We have extensive experience in grafting tissue recombinant of seminal vesicle and urogenital mesenchyme (from mouse or rat origin) that is handled as a solid

tissue piece. Since the fibroblasts are prepared as single cell suspensions, we needed to establish a new technique for generating tissue recombinants. This was previously described by Olumi *et al.*, *Cancer Research* 1999, but required some modifications to be successful in our hands.

Initially, we were concerned that all dispersed cells, suspended in the collagen gel, successfully made it under the kidney capsule site. Handling of the gel was difficult, especially whilst performing sub-renal grafting. We have been working hard on optimizing this technique over the past few months, in order to confirm that the collagen gel was an appropriate medium to transfer recombinants of dispersed cells under the kidney capsule.

Therefore, we set up tissue recombinants of dispersed rat seminal vesicle mesenchyme (rSVM)+hESC. We know from our previous work that rSVM (as a solid tissue piece) recombined with hESCs generates glandular tissue, similar to normal human prostate, that produces PSA (prostate-specific antigen) protein as detected by immunohistochemistry (Taylor *et al.*, *Nature Methods*, 2006). In order to confirm the same findings using the collagen gel transfer medium, we set up tissue recombinants of dispersed rSVM+hESC tissues and grafted them under the kidney capsule of SCID mice. The results from these grafts are shown below (Figure 2):

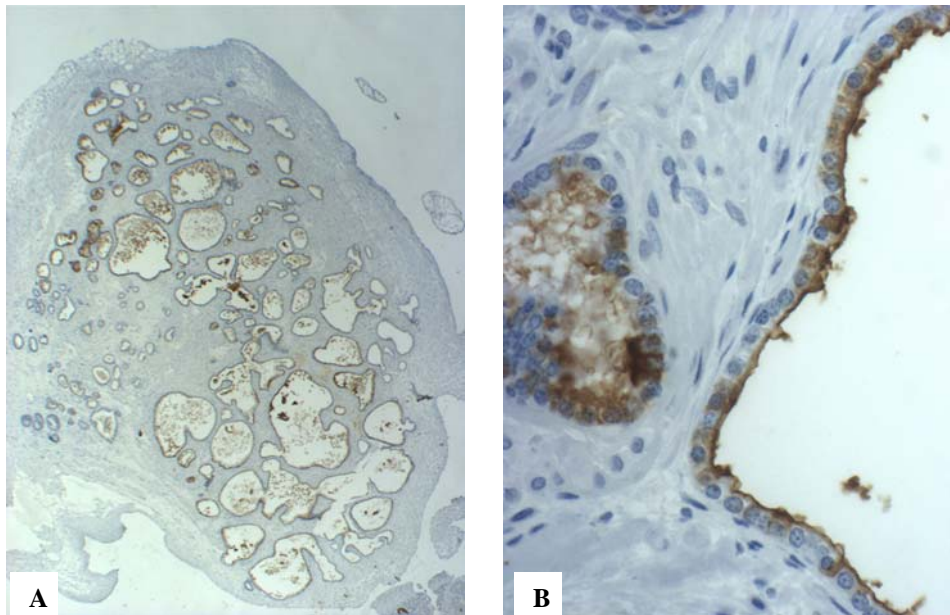


Figure 2: Prostate-specific antigen (PSA) immunolocalisation (brown stain) on tissue generated from dispersed rat seminal vesicle mesenchyme plus human embryonic stem cells in collagen gel (di-rSVM+hESC). Following 8 weeks in an immune-deficient (SCID) host mouse, glandular tissues were evident, with epithelial cells that produced human PSA (prostate-specific marker). A. Low power, B. High power.

Using this successful collagen gel technique, we recently set up a number of a second round of tissue recombinants using CAFs (of confirmed malignant phenotype; kindly provided by Assoc. Prof. Simon Hayward) are now being hosted in mice and will be collected in the coming weeks.

We generated a number of different combinations of cell types. Those combinations (and the current number of grafts hosted in mice) are listed in the table below.

Tissue Recombinants	Number of grafts	Result
CAF+hESC*	8	Grafts yet to be harvested
CAF alone*	3	Grafts yet to be harvested
NPF/hESC*	8	Grafts yet to be harvested
NPF/BPH-1*+	4	Grafts yet to be harvested
CAF/BPH-1*+	9	Grafts yet to be harvested

* Carcinoma-associated fibroblasts (CAFs; of confirmed pathology), normal prostatic fibroblasts (NPF) and BPH-1 cells kindly provided by Assoc. Prof. Simon Hayward, Vanderbilt University.

+ We included the NPF/BPH-1 and CAF/BPH-1 tissue recombinants as positive controls for these experiments. Previous studies by Hayward and colleagues describe these tissue recombinants; NPF/BPH-1 should result in benign prostate tissues, whilst CAF/BPH-1 should result in prostate carcinoma's (Hayward *et al.*, 2001, Grossfeld *et al.*, 1999).

These five tissue recombinations have been done using parental hES cell lines. The number of grafts in each group is low, and we will increase numbers, as well as test multiple time points in the next round of grafts. Once all those grafts are successfully harvested and analysed, we will repeat the studies with ENVY (GFP-expressing; Costa *et al.*, 2004) hESCs as mentioned in the previous annual report.

c. Measure wet weights and prepare tissues for analysis.

Analysis on the current tissue recombinants will begin in April 2006.

Task 3: Morphological analyses of tumorigenesis [Months 18-24]

- a. Immunohistochemistry for prostate specific antigen (PSA), androgen receptor (AR), cytokeratins (CK; including CK8, 18, 5, 14) and smooth muscle actin will show the extent of prostate differentiation and malignant transformation.**

No progress to date.

- b. Tumorigenic markers of cell proliferation (immunolocalisation of proliferating cell nuclear antigen; PCNA) and cell death (by apoptosis) will also be determined and quantitated.**

No progress to date.

KEY RESEARCH ACCOMPLISHMENTS

List of key research accomplishments emanating from this research:

- Obtained Human Ethics approval for the collection of human prostate tissues.
- Isolated fibroblasts from human prostate cancer patient samples using blind biopsy technique; mostly of benign pathology.
- Established new system for the collection of CAFs and NPFs in close collaboration with our pathologist; reliable method of isolating fibroblasts from regions of known pathology.
- Established cultured techniques of human prostatic fibroblasts.
- Established culture of hES 2 and hES 4 cells in the laboratory.
- Established protocol for collagen gel sub-renal grafting using some modifications to previous reports.
- Obtained CAFs and NPFs from Associate Professor Simon Hayward (Vanderbilt University).
- Generated several tissue recombinants consisting of CAFs, NPFs and hESCs and grafted under the kidney capsule of immune-deficient mice. Grafts yet to be harvested.
- Generated tissue recombinants using BPH-1 cells as positive control recombinants.

REPORTABLE OUTCOMES

	<i>Reportable outcomes that have resulted from this research:</i>
Manuscripts	<i>Manuscript by award recipient using similar techniques:</i> Taylor RA , Cowin PA, Cunha GR, Pera M, Trounson AO, Pedersen J, Risbridger GP (2006) Formation of human prostate tissue from embryonic stem cells. Nature Methods 3(3):179-181.
Abstracts Presentations	Speaker at 2 nd Pacific Rim Breast and Prostate Cancer Meeting, Palm Springs, CA (April 2005).
Patents and licences	Nil.
Degrees obtained	Nil.
Development of cell lines	Nil.
Tissue or serum repositories	Nil.
Informatics such as databases and animal models	Nil.
Funding applied for based on this work supported by this award	NH&MRC Peter Doherty Postdoctoral Training Fellowship.
Employment or research opportunities applied for and/or received based on experience/training supported by this award	Nil.

CONCLUSIONS

In summary, we have made significant progress towards establishing tissue recombinants composed of carcinoma-associated fibroblasts (CAFs) and human embryonic stem cells (hESCs).

Results from these experiments will reveal whether CAFs possess the ability to induce prostate carcinoma in undifferentiated embryonic stem cells.

Although we reported that we were close to collecting data at the end of last year, we experienced some problems with the cell types we were isolating. Since then, we have spent a good deal of time refining the protocols and techniques that allow these experiments to be completed with high reproducibility and confidence that the cells to be tested are of a malignant pathology. Therefore, when current recombinants are harvested we will be confident that the scientific findings are the direct result of CAFs initiation, and not due to anomalies in the methodology. We have also been thorough in setting up positive and negative control grafts to match the test conditions.

As reported last year, we are competently isolating and culturing human prostatic fibroblasts and human embryonic stem cells in the laboratory. In addition, we now have a reliable method of obtaining CAFs from prostate cancer patient tissues, with hands on involvement from our pathologist. We have been lucky to obtain CAFs and normal prostatic fibroblasts from our colleague to continue these experiments prior to our own system being established.

We look forward to the coming weeks when the current grafts will be harvested. Whether the CAFs are able to induce prostate carcinogenesis from hESCs or not, we are sure to contribute significantly to our understanding of the role of tumor stroma in the process of prostate carcinogenesis.

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